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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
The proposal tests the hypothesis that histone deacetylase activity contributes to the transcriptional repression of the methylated estrogen receptor α (ER) gene. It further postulates that inhibition of histone deacetylase (HDAC) and DNA methylation may act together to reactivate the ER gene. Studies to date show that the HDAC inhibitor trichostatin A, can reactivate ER expression in ER-negative breast cancer cell lines. Combined treatment with HDAC inhibitors and demethylating agents can synergistically reactivate expression of ER in ER-negative breast cancer cell lines.

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INTRODUCTION

This proposal tests the hypothesis that histone deacetylase activity contributes to the transcriptional repression of the methylated estrogen receptor α (ER) gene in ER-negative human breast cancer cells. It was further postulated that inhibition of histone deacetylation and DNA methylation may act together to reactivate ER expression.

BODY

Technical Objective 1: To determine whether histone deacetylase activity is required for transcriptional silencing of the ER gene.

As reported last year, studies showing that a histone deacetylase inhibitor can reactivate ER gene expression were completed and published in Yang et al, Cancer Research 60:6890-4, 2000.

Technical Objective 2: To achieve maximal ER reactivation both in vitro and in vivo through the combination of demethylation and histone deacetylase inhibition.

The combination of a histone deacetylase and a DNA methyltransferase inhibitor was shown to increase ER expression in a synergistic fashion when compared with either treatment alone. The combination led to a 300-400 fold induction in ER transcript. This was associated with ER protein re-expression. Restoration of estrogen responsiveness was demonstrated by the ability of the induced ER protein to elicit estrogen response element-regulated reporter activity from an exogenous plasmid as well as induce expression of the ER target gene, progesterone receptor. The synergistic activation of ER occurred concomitantly with markedly reduced soluble DNMT1 expression and activity, partial demethylation of the ER CpG island, and increased acetylation of histones H3 and H4. These data suggest that the activities of both DNMT1 and HDAC are key regulators of methylation-mediated ER gene silencing. These results were published in Yang et al, Cancer Research 61:7025-7029,2001. The animal studies proposed in the grant are in progress.

KEY RESEARCH ACCOMPLISHMENTS

Demonstration that the combination of an inhibitor of histone deacetylase and an inhibitor of DNA methyltransferase can synergistically reactivate ER expression in ER-negative human breast cancer cells over either strategy alone.

REPORTABLE OUTCOMES

Yang X, Phillips, DL, Ferguson AT, Nelson WG, Herman JG, and Davidson NE. Synergistic activation of functional estrogen receptor (ER)-α by DNA methyltransferase and histone deacetylase inhibition in human ER-α-negative breast cancer cells. Cancer Res 61:7025-7029, 2001.

As a result of her career development at Hopkins and through this grant, the original PI of this grant, Xiaowei Yang, M.D. Ph.D. took a position as a full-time permanent staff scientist at the National Cancer Institute, National Institutes of Health, Bethesda, MD in July, 2001. This grant was transferred to Yi Huang, M.D. Ph.D. who is completing this work and starting his own independent project.

CONCLUSIONS

Administration of a combination of histone deacetylase and DNA methyltransferase inhibitors can synergistically reactivate expression of ER in ER-negative breast cancer cell lines.

APPENDICES

One manuscript as listed above

[CANCER RESEARCH 61, 7025–7029, October 1, 2001]

Advances in Brief

Synergistic Activation of Functional Estrogen Receptor (ER)-α by DNA Methyltransferase and Histone Deacetylase Inhibition in Human ER-α-negative Breast Cancer Cells[1]

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Abstract

Formation of transcriptional repression complexes such as DNA methyltransferase (DNMT) 1/histone deacetylase (HDAC) or methyl-CpG binding protein/HDAC is emerging as an important mechanism in silencing a variety of methylated tissue-specific and imprinted genes. Our previous studies showed that treatment of estrogen receptor (ER)-α-negative human breast cancer cells with the DNMT inhibitor 5-aza-2′-deoxycytidine (5-aza-dC) led to ER mRNA and protein re-expression. Also, the HDAC inhibitor trichostatin A (TSA) could induce ER transcript about 5-fold. Here we show that 5-aza-dC alone induced ER transcript about 30–40-fold, and the addition of TSA elevated ER mRNA expression about 10-fold more in the human ER-negative breast cancer cell lines MDA-MB-231 and MDA-MB-435. Overall, the combination of 5-aza-dC and TSA induced a 300–400-fold increase in ER transcript. Restoration of estrogen responsiveness was demonstrated by the ability of the induced ER protein to elicit estrogen response element-regulated reporter activity from an exogenous plasmid as well as induce expression of the ER target gene, progesterone receptor. The synergistic activation of ER occurs concomitantly with markedly reduced soluble DNMT1 expression and activity, partial demethylation of the *ER* CpG island, and increased acetylation of histones H_3 and H_4. These data suggest that the activities of both DNMT1 and HDAC are key regulators of methylation-mediated *ER* gene silencing.

Introduction

ER[5] is a ligand-activated nuclear receptor that regulates transcription of estrogen-responsive genes in diverse target cells (1). ER and its ligand, 17β-estradiol, not only play a critical role in normal breast development but also have long been linked to mammary carcinogenesis, breast tumor progression, and outcome of breast cancer patients (2). Given the fact that 17β-estradiol stimulates the growth of ER-positive breast tumors via functional ER, endocrine therapy such as antiestrogen or ovarian ablation has been established as an important part of breast cancer management (3). However, up to one-third of breast carcinomas lack ER at the time of diagnosis, and a fraction of cancers that are initially ER positive lose ER during tumor progression (4). Genetic alterations, such as homozygous deletion, loss of heterozygosity, or *ER* gene mutation, have not been reported to play a major role in loss of ER expression. There is increasing evidence that epigenetic alterations play a role in inactivation of *ER* gene expression (5, 6). As demonstrated by Southern and MSP analyses, the *ER* CpG island is unmethylated in normal breast tissue and most ER-positive tumor cell lines, whereas it is methylated in ~50% of unselected primary breast cancers and most ER-negative breast cancer cell lines (7). The methylation of these CpG cluster sites is associated with either reduced or absent ER expression.

The DNMT inhibitor 5-aza-dC is widely used to study the reexpression of genes silenced by promoter methylation (8). 5-aza-dC exerts its demethylating function through sequestration of DNMT1 to 5-aza-dC-substituted DNA by the irreversible binding of the cysteine in the catalytic domain of the DNMT1 enzyme to the 6 position of the cytidine ring (9, 10). In our previous study, treatment of ER-negative human breast cancer cells with the methyltransferase inhibitors 5-aza-cytidine or 5-aza-dC led to partial demethylation of the ER CpG island, reexpression of ER mRNA, and synthesis of functional ER protein. Restoration of ER function was documented by eliciting ERE-driven promoter activity from an exogenous plasmid as well as the expression of the ER-responsive gene, *PR* (11).

Inactive chromatin built on methylated CpG clusters is emerging as an important molecular mechanism to silence a variety of methylated tissue-specific and imprinted genes (12, 13). This process involves methyl CpG-binding proteins to recruit HDAC family members. These HDACs remove acetyl groups from lysine residues of core histones, particularly H_3 and H_4, to increase ionic interactions between positively charged lysines of histones and negatively charged DNA, thereby generating a more compact nucleosome structure that limits gene activity. In our previous study, TSA, a potent and reversible HDAC inhibitor, could induce ER mRNA by 5-fold. This transcriptional activation is associated with increased sensitivity to DNase I at the *ER* locus without alteration of the methylated CpG sites, suggesting that an accessible chromatin structure is important in ER expression (6).

Recently, *in vitro* studies have shown that DNMT1 interacts physically with either HDAC1 or HDAC2 (14, 15), in addition to its ability to methylate hemimethylated CpG sites in DNA. These findings suggested that the ability of DNMT1 to repress transcription after replication through its regulatory NH_2 terminus is partially dependent on HDAC activity. A study using 5-aza-dC followed by TSA treatment of human colon cancer and leukemia cells robustly reactivated multiple methylated genes such as *MLH1*, *TIMP3*, *CDK2B*, and *CDK2A*. This finding supports the essential roles of both DNMT and HDAC in silencing expression of endogenous methylated genes (16).

Here we show that cotreatment with DNMT and HDAC inhibitors can synergistically induce *ER* gene expression in ER-negative breast cancer cells. Induced expression of ER mRNA and protein is associated with expected ER function on estrogen-responsive targets. First, ERE-driven reporter activity from an exogenous plasmid is induced by the treatments and blocked by the ER antagonist ICI 182,780. Secondly, the treatments stimulate expression of the ER-responsive

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[5] The abbreviations used are: ER, estrogen receptor; 5-aza-dC, 5-aza-2′-deoxycytidine; DNMT, DNA methyltransferase; ERE, estrogen response element; HDAC, histone deacetylase; MSP, methylation-specific PCR; PR, progesterone receptor; TSA, trichostatin A; PCNA, proliferating cell nuclear antigen; RT-PCR, reverse transcription-PCR.

gene, *PR*, which is again suppressed by ICI 182,780. Our data show that synergistic induction of ER occurs in conjunction with reduced soluble DNMT1 expression and DNMT activity, partial demethylation of the ER CpG island, and increased acetylation of histones H_3 and H_4. These data suggest that pharmacological intervention against both DNMT and HDAC can synergistically reactivate the methylated *ER* gene expression and restore ER function.

Materials and Methods

Cell Lines, Reagents, Antibodies, and Treatment Protocols. The human breast cancer cell lines (MDA-MB-231, MDA-MB-435, MCF-7/wt, and T-47D) and their culture conditions were as reported previously (17). Rabbit antihuman DNMT1 antisera were described previously (18). Specific anti-acetylated H_3 and H_4 rabbit polyclonal antibodies and PCNA monoclonal antibody were purchased from Upstate Biotechnology (Lake Placid, NY) and Oncogene Research Products (Cambridge, MA), respectively. The anti-ER-α antibody, 1D5, was obtained from Coulter Immunotech (Marseille, France). The pure antiestrogen ICI 182,780 was a generous gift from AstraZeneca Pharmaceuticals (Macclesfield, United Kingdom). For treatment, cells were seeded at a density of 5×10^5 cells/100-mm tissue culture dish. After 24 h of incubation, the culture media were changed to media containing 5-aza-dC for up to 96 h or TSA for 12 h. For the combination study, 5-aza-dC was present in culture for 96 h, and TSA was added for the last 12 h.

RNA Isolation, RT-PCR Analysis of ER and PR, and Quantitative Competitive PCR Analysis of ER. Total cellular RNA was extracted by Trizol reagent (Life Technologies, Inc., Rockville, MD). RT-PCR was carried out according to our previously described method (6). RNAs under comparison were simultaneously reversibly transcribed to achieve equal efficiency for reverse transcription. ER mRNA expression was initially screened by RT-PCR. If ER mRNA was present, a previously reported quantitative competitive PCR assay was performed to determine the level of ER transcript in treated MDA-MB-231 and MDA-MB-435 cells as compared with the expression level in untreated cells or the control ER-positive MCF-7 cells (6). PR RT-PCR primers were described previously (11), and the PCR reaction was performed with an Advantage GC2 PCR kit (Clontech, Palo Alto, CA) according to the protocol recommended by the supplier.

Genomic DNA Isolation and MSP Analysis of *ER* CpG Island. DNA was isolated by standard phenol-chloroform extraction. Isolated DNA was subjected to modification by sodium bisulfite to convert unmethylated cytosines but not methylated cytosines to uracils as described previously (19). Methylation status of the bisulfite-modified DNA at the *ER* locus was characterized by MSP using our previously reported method (7).

Western Blot Analysis of ER, PCNA, or DNMT1 Expression. Proteins from detergent-lysed cells were quantified by using the BCA protein assay kit (Pierce, Rockford, IL). The Western blot procedure was reported previously (20). Briefly, after protein separation by electrophoresis and transfer to nitrocellulose, blots were probed for 1 h with 2 μg/ml either mouse monoclonal antibody 1D5 (ER specific) or Ab-1 (PCNA specific) or affinity-purified rabbit polyclonal IgG (DNMT1 specific) at a 1:1000 dilution in PBS containing 5% dry milk. After incubation with peroxidase-labeled secondary rabbit antimouse or goat antirabbit antibodies, the immunoreactive proteins were detected by the enhanced chemiluminescence method as recommended by the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ). Protein band intensities were quantified by densitometry (EagleSight Software of Eagle Eye II Imaging System; Stratagene, La Jolla, CA).

DNMT Activity Assay. Lysates from cells growing in the presence or absence of 5-aza-dC, TSA, or 5-aza-dC and TSA were prepared and assayed for enzyme activity by the incorporation of [^{3}H]methyl-*S*-adenosylmethionine into poly(dI-dC:dI-dC) as described previously (21).

Analysis of Acetylated Histones. Cellular histone extraction was performed using 2×10^6 cells according to the procedure of Yoshida *et al.* (22), with the following modifications. The acid (H_2SO_4)-soluble supernatant was precipitated with 10 volumes of cold acetone. After overnight precipitation, histones were collected by centrifugation. The pellet containing histones was dissolved in 50 μl of H_2O, and proteins were quantified by using the BCA protein assay kit.

The extracted histones were separated by electrophoresis on a 15% SDS-PAGE gel and transferred to nitrocellulose membrane. After sequential incubations with rabbit anti-acetylated H_3 and H_4 polyclonal sera and goat antirabbit horseradish peroxidase-conjugated secondary antibody, the immunoreactive proteins were detected by the enhanced chemiluminescence method.

Transfections and Luciferase Assays. The ERE_2-tk-luciferase/SV-neo plasmid has been described previously (11). MDA-MB-231 cells were transfected with the plasmid using LipofectAMINE reagent and selected with G418 (1 mg/ml; Life Technologies, Inc.) for 2 weeks. Isolated colonies of G418-resistant cells were pooled and used for analysis. The pooled, transfected cells were maintained in culture media with 1 mg/ml G418. Luciferase activities were determined by Bright-Glo Luciferase Assay System (Promega, Madison, WI), and the activities were normalized for protein concentrations.

Results and Discussion

Induced ER Expression by DNMT and HDAC Inhibitors. The ER- and PR-negative cell line MDA-MB-231, which has densely methylated *ER* and *PR* CpG islands, was used as a cell model to test whether both DNMT and HDAC inhibition could synergistically reactivate ER expression. To test the hypothesis that pharmacological modulation of DNMT and HDAC by 5-aza-dC and TSA could synergistically activate *ER* gene expression, we first performed dose-response and time-course studies to characterize the effects of each individual drug. Maximal *ER* gene re-expression was achieved with 100 ng/ml TSA (0.33 μM) for 48 h or with 2.5 μM 5-aza-dC for 96 h (data not shown) when cells were treated with TSA or 5-aza-dC alone. Therefore, to test the hypothesis that combination treatment might have additive or synergistic effects, a treatment strategy of an optimal dose of 5-aza-dC (2.5 μM) for 96 h with the addition of TSA at 100 ng/ml for the last 12 h was used. We postulated that the reactivating effects of the demethylation agent may be potentiated by a short treatment with the HDAC inhibitor. Thus, for the combination studies, MDA-MB-231 cells were treated with 5-aza-dC for 96 h, and TSA was added for the last 12 h. As shown in Fig. 1*A*, ER mRNA was induced 31-fold by treatment with 5-aza-dC for 96 h as compared with vehicle-treated cells, whereas treatment with TSA alone for 12 h had little effect. The combined treatment led to a ~400-fold induction in ER transcript. Thus, the brief addition of TSA to optimal 5-aza-dC treatment increased ER mRNA by about 13-fold, suggesting a synergistic effect.

To ascertain whether both DNMT and HDAC activities could play a role in repression of ER expression more generally, the dual treatment strategy was evaluated in another ER-negative human breast cancer cell line, MDA-MB-435. Optimal *ER* gene re-expression was observed after treatment of these cells with either 50 ng/ml (0.165 μM) TSA for 48 h or 0.6 μM 5-aza-dC for 96 h when these drugs were used as single agents (data not shown). The same combination treatment used for MDA-MB-231 cells was used for MDA-MB-435 cells. As shown in Fig. 1*B*, treatment with 5-aza-dC alone induced ER mRNA by 36-fold, whereas treatment with TSA alone for 12 h had little effect. The combined treatment led to a 318-fold induction of ER transcript compared with that of vehicle-treated MDA-MB-435 cells (Fig. 1*B*). Thus, two different ER-negative cell lines showed evidence of synergistic reactivation of ER by the combined approach of DNMT and HDAC inhibitors.

We next studied how the levels of ER reactivation compared with endogenous ER expression in the human breast cancer cell line MCF-7. As shown in Fig. 1*C*, 5-aza-dC induced ER transcripts in MDA-MB-231 and MDA-MB-435 cells to 4% and 2%, respectively, of the expression level found in MCF-7 cells. The addition of TSA to the 5-aza-dC-treated cultures induced ER mRNA to 50% and 20% of the MCF-7 expression levels, respectively. Thus, the combination treatment consistently and synergistically induced ER transcript in ER-negative breast cancer cell lines, supporting our hypothesis that

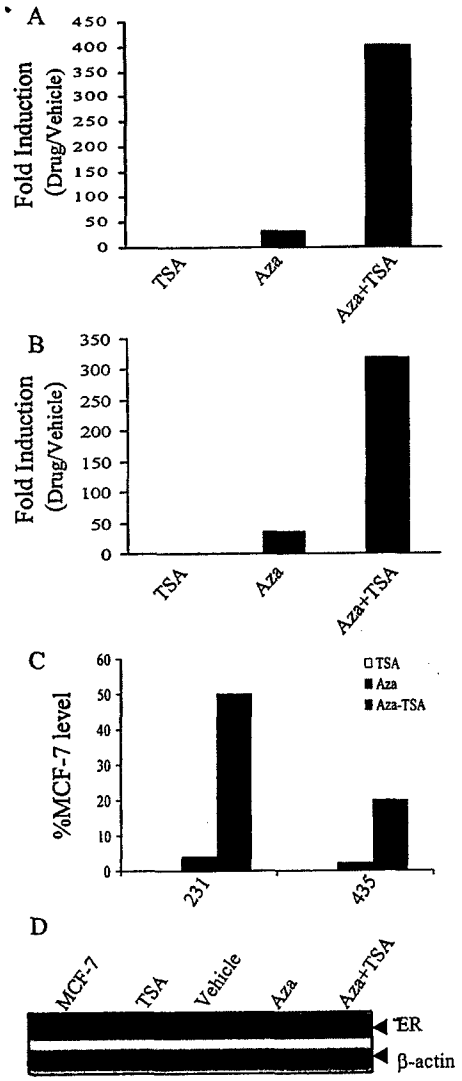

Fig. 1. Effects of DNMT and HDAC inhibitors on ER expression in ER-negative breast cancer cells. *A* and *B*, effects of TSA, 5-aza-dC, or the combination of both on ER transcript, as detected by a quantitative competitive PCR. The ratio of ER mRNA (drug-treated cells/vehicle-treated cells) is designated as fold induction (see "Materials and Methods") in MDA-MB-231 (*A*) and MDA-MB-435 (*B*). A representative example of three experiments that gave similar results is shown. *Aza*, 5-aza-dC. *C*, relative levels of ER activation in drug-treated cells compared with the level of ER mRNA in MCF-7 cells. *231*, MDA-MB-231; *435*, MDA-MB-435. *D*, Western blot analysis of ER protein expression from nuclei-enriched fraction of proteins (50 μg/lane) in drug-treated and vehicle-treated MDA-MB-231 cells. Whole cell lysates from MCF-7 cells were used as an ER protein-positive control. A representative example of two independent experiments that gave similar results is presented. β-Actin was probed as a protein loading control.

the activities of both DNMT and HDAC are key regulators in *ER* gene silencing. The synergistic effect with an optimal exposure to DNMT inhibitor and a suboptimal exposure to HDAC inhibitor suggested a layered action on ER activation.

However, the combination of DNMT and HDAC inhibitors apparently did not reactivate ER mRNA expression to the levels seen in MCF-7 cells. This finding could reflect experimental variation or differences in genotypes between cell lines. Alternatively, it is possible that other mechanisms might also play a role in the silencing process. For example, other transcriptionally repressive complexes such as methyl-CpG binding protein/HDAC could also be involved in the silencing process. Also, some corepressor proteins in various repressive complexes are partially HDAC independent. For instance, a component of the repression mediated by the MeCP2 transcriptional repression domain is partially HDAC independent, and mSin3A could retain some ability to repress transcription, even in the absence of associated HDACs (12). Also, DMAP-1, a corepressor in the DNMT1/HDAC gene transcriptional repression complex, is HDAC independent (14). Therefore, simultaneous inhibition of several components in the methylation-associated repressive complexes might be necessary to achieve maximal reactivation of repressed genes.

This effect appeared to be relatively specific for the methylated *ER* gene because the treatments (alone or in combination) had no apparent effect on the level of expression of the unmethylated housekeeping gene, β-actin, using a competitive quantitative PCR assay (data not shown). However, the combined treatment of the ER-negative breast cancer cells did reactivate expression of other genes silenced by methylation such as RARβ2 or cyclin D2 (data not shown) in addition to ER.

Next, we investigated whether the activated ER transcript was translated into ER protein by Western analysis. The ER protein signal in 5-aza-dC-treated MDA-MB-231 cells was compared with that in the combination-treated cells after normalizing with β-actin protein. As shown in Fig. 1*D*, 5-aza-dC treatment of MDA-MB-231 cells reactivated the M_r 67,000 ER protein, whereas TSA had little effect. The amount of ER protein in combination-treated cells was about twice that in cells treated with 5-aza-dC alone. These data provide further evidence that *ER* gene expression was enhanced by the combined treatment with 5-aza-dC and TSA as compared with that seen with either drug alone.

Analyses of Activated ER Function in MDA-MB-231 Cells. The ability of the re-expressed ER protein to mediate an estrogen response was tested next. For this study, a plasmid that contains a firefly luciferase gene under the transcriptional regulation of two EREs driving a thymidine kinase promoter was stably transfected into MDA-MB-231 cells. These cells were treated with 5-aza-dC or TSA alone and with the combination of both (see "Materials and Methods") in estrogen-containing culture media. As shown in Fig. 2*A*, treatment

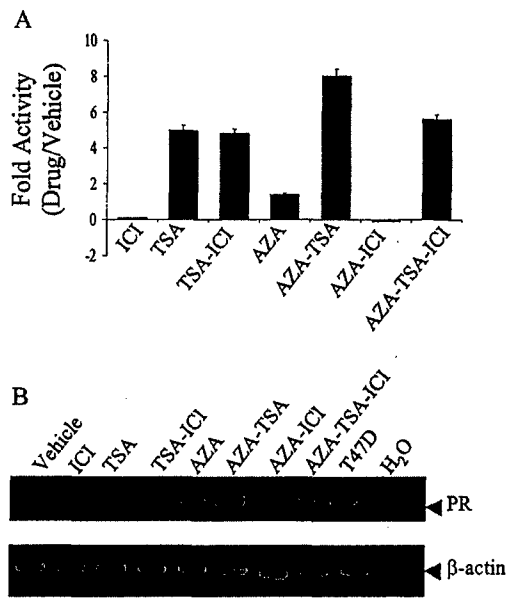

Fig. 2. Effects of DNMT or HDAC inhibition on ERE-driven promoter activity and endogenous estrogen-responsive *PR* gene transcription in MDA-MB-231 cells. *A*, effects of treatments on ERE-regulated reporter activity in MDA-MB-231 cells stably transfected with ERE_2-tk-luc/SV-neo plasmid. Fold activity is calculated from the relative luciferase values in drug-treated *versus* vehicle-treated cells in triplicate samples after the luciferase units were adjusted for the protein concentration in each setting (mean fold activity ± SD). A result from one of three independent experiments that give similar results is shown. *B*, effects of treatment on *PR* gene expression. *Top panel*, the predicted 400-bp PR transcript expression in MDA-MB-231 cells. A representative example of four experiments with similar results is shown. T-47D was used as a PR PCR-positive control, and H_20 was used as a PCR-negative control. *AZA*, 5-aza-dC; *ICI*, ICI 182, 780. *Bottom panel*, β-actin RT-PCR product provides a control for the amount of intact RNA used in the reaction.

with 5-aza-dC alone led to a 1.4-fold increase in promoter activity. TSA alone induced the reporter activity by 5-fold, an unexpected finding because 12 h of TSA induced little ER mRNA or protein. Possibly, this is due to the chromatin remodeling effect of TSA instead of ER involvement. Nevertheless, cotreatment with 5-aza-dC and TSA elicited a more than additive induction of reporter activity (about 8-fold; Fig. 2*A*). Thus, the combination treatment enhanced ERE-driven reporter transcription in MDA-MB-231 cells when compared with single agents.

To confirm that drug-activated reporter activity was a specific estrogen effect mediated through the action of the activated ER, the ER antagonist ICI 182,780 (5 μM) was used to block estrogen effects in the culture (23). As shown in Fig. 2*A*, ICI 182,780 suppressed 5-aza-dC-stimulated ERE-mediated reporter activity but had no effect on TSA-mediated transcription. These results indicate that 5-aza-dC-activated reporter transcription (5-aza-dC alone or the combination) is a specific hormone effect and suggest that the effect of treatment by TSA alone on this reporter activity is not completely related to estrogen response. Indeed, the exact nature of the effect of TSA on this reporter needs to be further explored.

We further investigated the ability of the drug-induced ER to activate expression of the endogenous ER-responsive gene, *PR*. As shown in Fig. 2*B*, treatment of MDA-MB-231 cells with 5-aza-dC or the combination induced PR mRNA expression, whereas ICI 182,780 or TSA alone had little or no effect. Cotreatment of MDA-MB-231 cells with 5-aza-dC/ICI 182,780 suppressed PR expression. Similarly ICI 182,780 treatment of cells growing in 5-aza-dC and TSA led to a marked decrease in PR expression. Together, these data suggest that drug-induced ER is functionally active.

Changes in DNMT1 Expression, ER CpG Island Methylation, and Increased H_3 and H_4 Acetylation Introduced by DNMT and HDAC Inhibitors. To study the underlying mechanisms associated with the synergistic activation of the methylated *ER* gene, we examined the expression and activity of the methylation maintenance enzyme, DNMT1. The results in Fig. 3*A* showed that addition of 2.5 μM 5-aza-dC to the culture of MDA-MB-231 cells for 96 h markedly depleted soluble DNMT1 protein expression and total DNMT activity from the whole cell protein lysates (Fig. 3*B*). Interestingly, TSA also down-regulated soluble DNMT1 protein expression with some reduction in DNMT activity. Down-regulation of DNMT1 expression by TSA was consistently obtained in several independent experiments. The combination treatment further reduced the amount of DNMT1 protein and total DNMT activity. Although the effect of 5-aza-dC on soluble DNMT1 protein and total DNMT activity was expected because it sequesters DNMTs after its incorporation into genomic DNA and inhibits its enzyme activity (10), the effect of TSA was not. One possible explanation is that the effect of TSA on DNMT1 expression and DNMT activity reflects its ultimate antiproliferative effects. A second explanation is that a possible functional or physical interaction exists between DNMT1 and HDAC. Because the cell proliferation marker PCNA interacts physically with DNMT1 (24), we examined its expression by immunoblot. As shown in Fig. 3*A*, PCNA expression was not obviously modulated by any treatment. Thus, our data suggest a possible functional connection between DNMT1 and HDAC or physical dependency for protein stabilities because no obvious down-regulation of DNMT transcript was observed by the various treatments (data not shown). However, the discrepancy between the level of DNMT1 depletion and reduction in total DNMT activity induced by TSA could also reflect a role of other DNMT family members that are not affected by TSA.

Next, the MSP assay was used to examine the methylation status of the *ER* CpG island in MDA-MB-231 cells after the treatments (Fig. 3*C*). The *ER* CpG island remained methylated in MDA-MB-231 cells

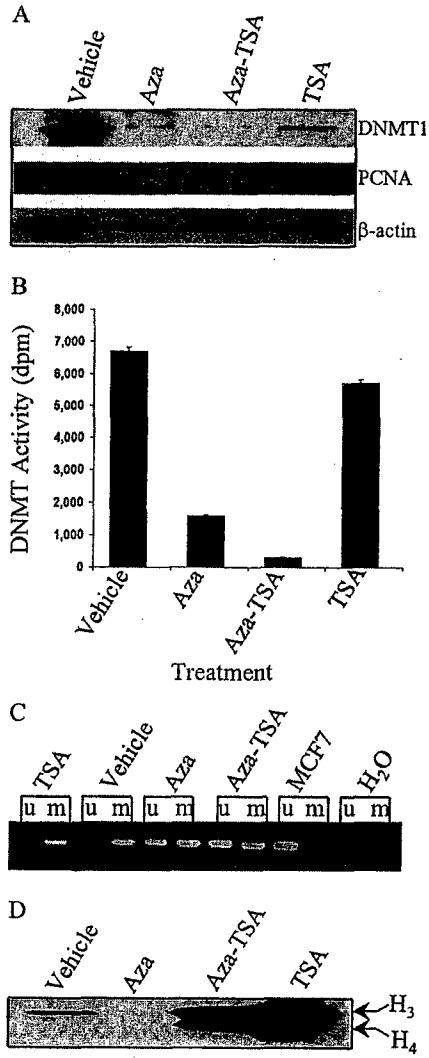

Fig. 3. Effects of inhibitors of DNMT and HDAC on soluble DNMT1 expression and DNMT activity, ER CpG island methylation, and the acetylation of histones H_3 and H_4 after the treatments (see "Materials and Methods"). *A*, DNMT1 and PCNA expression levels were analyzed by Western blot analysis. Equal amounts of MDA-MB-231 protein lysates (50 μg/each lane) from drug-treated and vehicle-treated cells were subjected to analysis. The blot was reprobed with anti-β-actin antibody as a protein loading control. A representative result of three independent experiments is presented. *B*, analysis of DNMT activity. Cell lysates from MDA-MB-231 cells grown in the presence or absence of 5-aza-dC, TSA, or 5-aza-dC and TSA were assayed for enzyme activity. Data are presented as the mean dpm ± SD of triplicate samples. *C*, MSP analysis of *ER* CpG island methylation pattern using a representative primer set ER5 after the treatments. ER-positive MCF-7 was used as an unmethylated control, untreated MDA-MB-231 cells were used as a methylated control, and H_2O was used as a MSP-negative control. *Lanes m*, methylated products; *Lanes u*, unmethylated products. *D*, histone H_3 and H_4 acetylation profiles in MDA-MB-231 cells after the treatments (see "Materials and Methods"). Western blot analysis of equal amounts of total acid-extracted proteins (50 μg/lane) from drug-treated and vehicle-treated MDA-MB-231 cells using specific anti-acetylated H_3 and H_4 antibodies is shown.

treated with vehicle or TSA (100 ng/ml for 12 h). However, treatment with 5-aza-dC alone or in combination with TSA led to partial demethylation of the CpG island, in conjunction with *ER* gene re-expression. These data are consistent with our previous findings and reaffirm that DNA methylation is a participant in the regulation of *ER* gene expression (11).

We then studied whether HDAC inhibition directly acetylates histones and whether the increased histone acetylation is associated with *ER* gene activation. As shown in Fig. 3*D*, the HDAC inhibitor TSA up-regulated H_3 and H_4 acetylation as expected, although the magnitude of *ER* gene activation was not clearly proportional to the degree of histone H_3 and H_4 acetylation.

In summary, a demethylated ER promoter sequence with increased histone H_3 and H_4 acetylation by sequential inhibition of both DNMT and HDAC is correlated with the synergistic activation of the *ER* gene. These data indicate that pharmacological intervention against DNMT and HDAC can lead to alteration of *ER* gene methylation and chromatin conformation change characterized by hyperacetylated histones. Future studies using coimmunoprecipitation or chromatin-immunoprecipitation assay to elucidate the individual components that form the transcriptionally repressive complex at the *ER* gene locus will be pursued, and the effects of pharmacological manipulation will be further explored.

Because ER is a critical growth-regulatory gene in breast cancer, it is important to better understand its transcriptional regulation. Our data suggest that a combination drug regimen of both DNMT and HDAC inhibitors can synergistically activate functional ER protein. Additional studies will be necessary to evaluate the clinical implications of this finding with regard to sensitivity to selective ER modulators such as tamoxifen or ICI 182,780.

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